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Original article

Genetic diversity and phylogenetic relationship among the western and the Asian honey bees based on two mitochondrial gene segments (COI and ND5)

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ABSTRACT

The Asian honey bee species i.e., *Apis cerana* (the eastern honey bee), *A. dorsata* (the giant honey bee), and the western or European honey bee (*A. mellifera*) collected from Pakistan were studied using partial sequences from two mitochondrial genes (i) the Cytochrome c oxidase I (COI) and (ii) the mitochondrially encoded NADH dehydrogenase 5 (ND5) and then compared with other honey bees sequences (already submitted from different countries around the globe) obtained after the national center for biotechnology information (NCBI). DNA sequences were analyzed employing molecular evolutionary genetics analysis and Kimura 2-parameter model, neighbor-joining method was applied to investigate phylogenetic relationships, and DNA sequence polymorphism was applied to measure the genetic diversity within the genus *Apis*. The phylogenetic analyses yielded consistent results. Based on COI gene fragment in two Asian and European honey bee species from Pakistan and from other countries showed considerable genetic diversity levels and deviation among the species. While in contrast the phylogenetic analyses based on ND5 gene fragment in Asian and European honey bee species from Pakistan and other countries showed comparatively higher genetic diversity indices and variations than the COI gene. So, in the genus *Apis*, the mitochondrial ND5 region has shown the possibility to answer the interactions among species. A further detailed work (by linking the analysis of other genomic and mitochondrial genes) is required for good quality solution to establish the concise genetic diversity and interaction among the *Apis* species. The objective of this study was to explore the extent of genetic differences and phylogenetic links among the three kinds of honey bee species from Pakistan and comparing them with other bee species around the globe.

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1. Introduction

The classification of honey bees (*Apis* spp.) is important because of their economical (source of honey and bee products), nutritional, and ecological role (the bees pollinate wide varieties of crops and

wild vegetation). Currently, the genus *Apis* has 10 commonly identified species i.e. *A. andreniformis*, *A. binghami*, *A. cerana*, *A. dorsata*, *A. florea*, *A. koschevnikovi*, *A. laboriosa*, *A. mellifera*, *A. nuluensis*, and *A. nigrocincta* (Arias and Sheppard, 2005). Based on the limited difference on morphological characters some species have been questioned e.g., *A. laboriosa* does not have enough morphological characters to be distinguished from *A. dorsata* (Koeniger and Koeniger, 1991; Engel, 1999) but both these have markable difference in performance and ecosystem (McEvoy and Underwood, 1988; Kirchner et al., 1996; Otis, 1996). The distribution of western honey bee, *A. mellifera* is an allopatric and its original habitat is Europe, Africa, and central Asia while the distribution of all other species of the genus *Apis* is Asia and these species are sympatric in tropical regions (Arias and Sheppard, 2005).

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By using various molecular marker techniques, the genetic variety and gene pool of insects may be studied better and questions on distinguishing species based on morphometric characters can be answered more clearly. Mitochondrial DNA, random amplified polymorphic DNA, microsatellites, expressed sequence tags, and amplified fragment length polymorphism markers are important molecular techniques which are being employed in insect ecological studies and have contributed significantly in understanding the genetic origin of insect variety (Behura, 2006). Bees are mostly classified through their morphological characters, but their genetic variations are observed through mitochondrial DNA gene segments (Branchiccela et al., 2014; Ostroverkhova et al., 2015), single nucleotide polymorphism (Chapman et al., 2016), and allozymes (Smith and Glenn, 1995) to differentiate the races between the species. The molecular analysis offers a compelling tactic to measure the extent of genetic difference between and among the honey bee species (Meemongkolkiat et al., 2019).

In molecular markers, the mitochondrial genes are commonly used in insects to analyze the extent of genetic diversity (Bouga et al., 2011) since ten times high average mutation rates are present in the mitochondrial genome in comparison to the nuclear genome (Ballard and Whitlock, 2004) owing to nucleotide imbalance (Song et al., 2003) and the insignificant effectual population size linked to active haploid heritage among eukaryotic taxa (Neiman and Taylor, 2009). The polyandrous mating approach in honey bees and other eusocial insects shows to introduce vast substitution ratios in mitochondrial genes as compared to nuclear genes because organisms inherit their mitochondrial DNA solely from the maternal lineage (Meusel and Moritz, 1993).

In Pakistan, total four species of the genus *Apis* are found. Out of these four species, three are native i.e., *A. cerana*, *A. dorsata*, *A. florea* while *A. mellifera* is imported from Europe (Khan, 2020; Sajid et al., 2020; Shakeel et al., 2020). There is very scant literature on morphological and molecular studies on the Asian and the western honey bee species found in the country. Although some molecular studies of *A. mellifera* were conducted based on mitochondrial gene segment i.e., cytochrome *c* oxidase I (Rizwan et al., 2018) but no molecular data about other Asian honey bee species are present.

So, the study was designed to better explore the extent of the genetic difference and phylogenetic interactions among three kinds of honey bee species (*A. cerana*, *A. dorsata*, and *A. mellifera*) from Pakistan and comparing them with additional honey bee species around the globe using partial sequence data from two mito-

chondrial genes (COI and ND5). The partial sequences of COI and ND5 were successfully amplified from the Asian and the western honey bee species and the ND5 sequences of *A. cerana*, *A. dorsata*, and *A. mellifera* were made for the first time from Pakistan. The data will offer balancing evidence and adequate knowledge of the phylogenetic difference and the basis among the genus *Apis*.

2. Materials and methods

2.1. Specimen collection, DNA extraction, PCR amplification, and sequencing

The Asian honey bees (*A. cerana* and *A. dorsata*) were collected from wild colonies, while the European honey bees (*A. mellifera*) were gathered from managed colonies (Table 1). These specimens were moved to the research laboratory in pure ethanol and were preserved at -80°C while waiting for further use. The entire DNA was obtained from the thorax of honey bees applying QIAamp DNA Mini Kit (Qiagen, Hilden-Germany) in line with the company's procedure and analyzed through agarose gel electrophoresis. Partial regions of two mtDNA genes (COI and ND5) were augmented by using a Gene Amp PCR System 9700 (Applied Bio systems). Polymerase chain reaction (PCR) was done applying the primers mentioned in table 2. All ND5 primers were created using a tool on the NCBI website. PCR reactions consisted of a total volume of 50 μL reaction mixture containing 26 μL of Taq PCR Master Mix (Qiagen), 2 μL of every primer (forward and reverse primer each with a concentration of 10 μM), 10 μL of DNA template (concentration $> 30 \text{ ng}/\mu\text{L}$), and 10 μL of nuclease free water. PCR package entailed an early denaturation step started at 95°C for 4 min, subsequently 25 drives of denaturation 95°C used for 20 sec, annealing on 53°C intended for 45 sec, and elongation by 72°C meant for 90 sec, and a last phase of elongation by 72°C designed for 10 min. The amplified PCR products were studied by gel electrophoresis (U: GENIUS) in 1.2% agarose gel in 1.5 X TBE buffer (120 V, 35 min.), and visualized in a gel system (Clever Scientific Ltd., UK) by staining through ethidium bromide then filtered with GeneJET Gel Extraction Kit (ThermoFisher Scientific, OH, USA) consistent with the provider's procedure. Next to the refinement of PCR products, the entire samples were dispatched to commercial sequencing MacroGen Firm for two-way sequencing. Separate sequences were revealed through computerized sequencing of both strands of every COI and ND5 gene section make using BioEdit

Table 1

The details of collection sites, coordinates, and country of collected honey bee specimens.

Honey bee species	Specimen Code	Collection site	Country	Coordinates
<i>Apis cerana</i>	HB-11	Islamabad	Pakistan	33.779755, 73.242013
<i>A. cerana</i>	HB-27	Murree	Pakistan	33.917139, 73.392352
<i>A. dorsata</i>	HB-20	Rawalpindi	Pakistan	33.568782, 73.082827
<i>A. mellifera</i>	HB-14	Rawalpindi	Pakistan	33.652272, 73.079365

Table 2

The detail of primers applied for the amplification of mitochondrial gene segment. (CO I and ND5).

S. No.	Target gene	Primer name	Sequence	Honey bee species	Reference
1	COI	LC01490 HCO2198	5'-GGTCAACAAATCATAAAGATATTGG-3' 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'	AM, AC, AD	(Vrijenhoek, 1994)
2	ND5	ND51_F ND51_R	5'-ATTCAAAATTTGCAACTAAACCA-3' 3'-ATTGATTGTGGGATCAATG-5'	AM	Designed
3	ND5	ND52_F ND52_R	5'-GAAACTGTAAAAATAGTTCCAACA-3' 3'-GGATGAGATGTTTAGGATT-5'	AC	Designed
4	ND5	ND54_F ND54_R	5'-ATGTTGAATAAGCAACAACCT-3' 3'-GGTTGAGATGGATTAGGATTA-5'	AD	Designed

COI = Cytochrome *c* oxidase I; ND5 = mitochondrially encoded NADH dehydrogenase 5; AM = *Apis mellifera*; AC = *A. cerana*; AD = *A. dorsata*.

7 (Hall, 1999; Khan et al., 2017). All the sequences were allied employing Clustal X (Thompson et al., 1997) and then aligned by hand. Distinctive COI and ND5 sequences of the entire samples were banked at the NCBI GenBank database by accession numbers (MW888444; MW888445; MW888446; MW888447; MW889003; MW889004; MZ004848).

2.2. Data and phylogenetic analysis

For COI sequences of *A. mellifera*, 23 sequences were obtained from the NCBI database while a single sequence was obtained from the Pakistani sample, sequenced, deposited to NCBI GenBank, and its accession number was obtained. For ND5 sequences of *A. mellifera*, 22 sequences were obtained from the NCBI database while one sequence was obtained from a Pakistani sample, sequenced, deposited to NCBI GenBank, and its accession number was obtained. While, for COI sequences of *A. cerana*, 20 sequences were obtained from the NCBI database while one sequence was obtained from a Pakistani sample, sequenced, deposited to the NCBI GenBank, and its accession number was obtained. For ND5 sequences of *A. cerana*, 13 sequences were obtained from NCBI database while one sequence was obtained from a Pakistani sample, sequenced, deposited to the NCBI GenBank, and its accession number was obtained. Similarly, for COI sequences of *A. dorsata*, 18 sequences were obtained from the NCBI database while one sequence was obtained from Pakistani sample, sequenced, deposited to the NCBI GenBank, and its accession number was obtained. For ND5 sequences of *A. dorsata*, 2 sequences were obtained from the NCBI database while one sequence was obtained from Pakistani sample, sequenced, deposited to NCBI GenBank, and its accession number was obtained.

MEGA-X was used to perform multiple-sequence alignments with MUSCLE by utilizing the default factors, and then the computer originated alignments were more modified manually. Moreover, the alignments of the COI and ND5 data sets were validated compared to their respective sequences of *A. mellifera*, *A. cerana*, and *A. dorsata* samples available in GenBank. Likewise, pairwise hereditary differences were assessed employing MEGA X (Kumar et al., 2018) and the Kimura 2-parameter model (Kimura, 1980). Neighbor-joining (NJ) examinations were performed on each gene portion upon the interconnected data set. The NJ evaluation was conducted using the Kimura 2-parameter model (Kimura, 1980) with MEGA X (Kumar et al., 2018) for the whole data sets. Trust in nodes was estimated by 1000 bootstrap repeats (Felsenstein, 1985). For the construction of the phylogenetic trees, we used a butterfly *Afrota ganga* sequences taken from Genbank (accession number: KF590536), as an out group so as to root the trees.

The portion (1 bp to 671 bp) of the COI gene sequence of *A. mellifera* applied in the study occurred amid the nucleotide 6–676 bp (Hymenoptera sp. BT-254 COI gene, partial cds; mitochondrial 685 bp, Accession: MK029969). The portion (1 bp to 476 bp) of the COI gene sequence of *A. cerana* used in this study fall among nucleotide 1931–2406 bp of *A. cerana* mitochondrion, complete genome, Accession: GQ162109, the total sequence length of 15895 bp (Tan et al., 2011). The portion (2 bp to 654 bp) of the COI gene sequence of *A. dorsata* used in the evaluation was between nucleotide 1888–2540 bp (*A. dorsata* mitochondrion, complete genome, Accession: NC_037709).

The portion (1 bp to 743 bp) of the ND5 gene sequence of *A. mellifera* employed occurred amongst nucleotide 8226–8968 bp of *A. mellifera sinixinyuan* mitochondrion, complete genome, Accession: MN733955.1, the total sequence length of 16886 bp. The portion (1 bp to 474 bp) of the ND5 gene sequence of *A. cerana* was in the middle of nucleotide 7594–8067 bp of *A. cerana* mitochondrion, complete genome Accession: KX908206.1, the total sequence length of 15904 bp. The portion (1 bp to 441 bp) of the

ND5 gene sequence of *A. dorsata* occurred among nucleotide 7550–7990 bp of *A. dorsata* mitochondrial DNA, complete genome Accession: AP018369, total sequence length 15279.

2.3. Genetic diversity

Mitochondrial diversity based on two gene segments among three honey bee species, all indices were performed in DNA sequence polymorphism (Dna SP v5.10 (Librado and Rozas, 2009) underneath two neutrality examinations Fu and Li's F^* (Fu, 1997) and Tajima's D (Tajima, 1989).

3. Results

Three honey bee species were gathered from various places of Pakistan. A total of 24, 21, and 19 COI sequences and 23, 14, and 03 ND5 sequences (obtained from this study and downloaded from the NCBI website) of *A. mellifera*, *A. cerana*, and *A. dorsata* honey bee species respectively were blasted to ascertain the resemblance index and recognition of every isolate from Pakistan and other countries are displayed in the phylogenetic trees. For mitochondrial COI gene fragment, there was 97.76–99.85% (total score 1157–1234; query cover = 97–100%; E value = 0.0–0.0), 91.18–95.17% (total score 647–752; query cover = 92–100%; E value = 0.0–0.0), and 93.34–99.85% (total score 935–1203; query cover = 80–100%; E value = 0.0–0.0) nucleotide identity for *A. mellifera*, *A. cerana*, and *A. dorsata* sequences correspondingly when these sequences were blasted separately in the NCBI database.

For mitochondrial ND5 gene fragment, for *A. mellifera*, 82 hits were found in the NCBI database with the nucleotide identity of 99.33%–97.85% (total score 1290–1345; query cover = 100–100%; E value = 0.0–0.0). For *A. cerana*, only 12 hits were found in the NCBI database with a nucleotide identity of 94.55%–99.79% (total score 734–870; query cover = 99%–100%; E value = 0.0–0.0). Similarly, for *A. dorsata*, only two hits were found in the NCBI database with a nucleotide identity of 97.96–98.19% (total score 765–771; query cover = 100–100%; E value = 0.0–0.0).

3.1. Phylogenetic relationship among honey bee species

The evolutionary relationship of honey bee species based on two mitochondrial gene fragments (COI and ND5) was inferred. A phylogenetic tree based on COI gene fragment is shown in Fig. 1. This tree categorized the whole assemblage of *A. mellifera*, *A. dorsata*, and *A. cerana* into three main groups from different countries, including Pakistan into three main groups. *A. mellifera* had three further sub groups first one consisted of species belonging to the USA, European countries, and China. The second group consisted of species mostly distributed in African. In contrast, the third group consisted of species from Saudi Arabia only. *A. mellifera* sequence of Pakistan fell into the first group and was closer to the specimen (KT074028) from the United Kingdom. *A. dorsata* had a single group that consisted of species from different Asian countries. *A. dorsata* sequence of Pakistan was closely related to two specimens i.e., one specimen (KT960840) from India and another Pakistani specimen (KT835209). The *A. cerana* species also formed a single group but few species from India and Japan fall separately at larger distances. *A. cerana* sequence of Pakistan was also fall separately and closely related to a specimen (AP017984) from Japan.

A phylogenetic tree based on ND5 gene fragment is shown in Fig. 2. This tree also put together the complete compilation of *A. mellifera*, *A. dorsata*, and *A. cerana* from different countries including Pakistan. *A. mellifera* had three further sub groups. The first subgroup was separated into two divisions. One consisted of species belonging to the USA and European countries, while the sec-

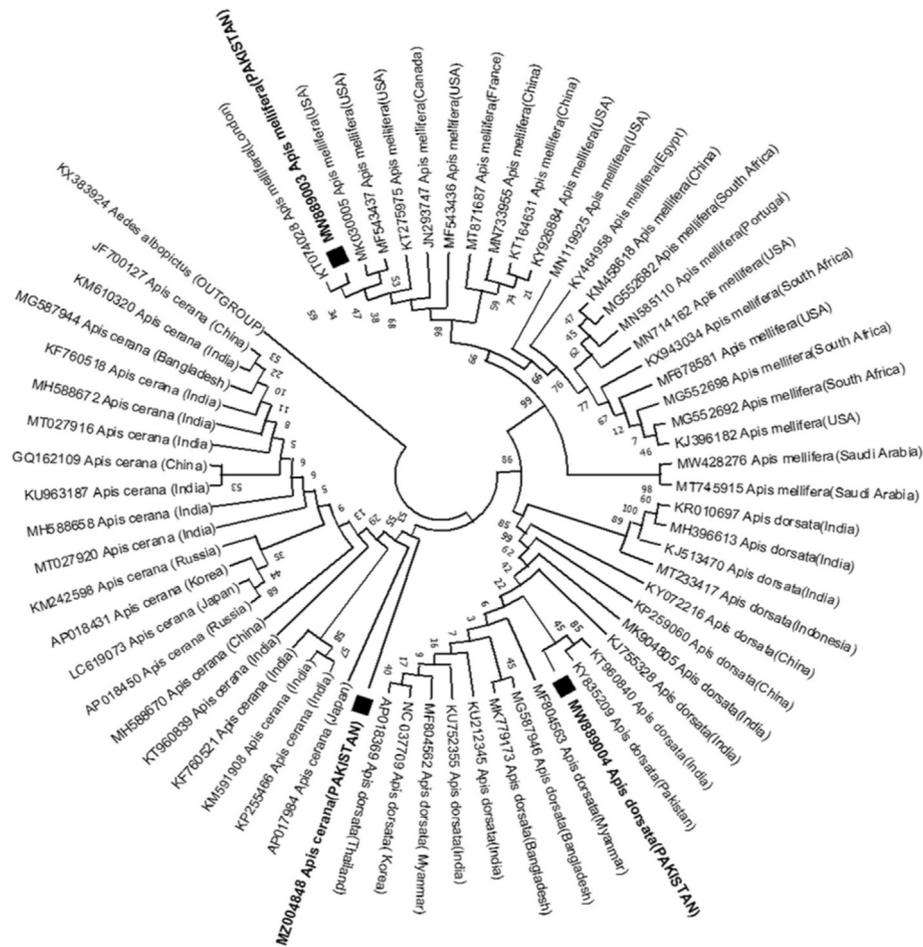


Fig. 1. The evolutionary description was assumed employing the Neighbor-Joining method (Saitou and Nei, 1987). The best possible hierarchy by the totality of branch size = 0.97583741 is displayed. The proportion of repeated hierarchy where the correlated taxa grouped collectively in the bootstrap test (1,000 replicates) are exhibited alongside the branches (Saitou and Nei, 1987). The evolutionary spaces were processed employing the Kimura 2-parameter method (Kimura, 1980) and remain here the unit of the quantity of base substitutions per site. The assessment engaged 65 nucleotide sequences. Codon positions contained were first + second + third + Noncoding. The whole vague locations were deleted for every sequence pair (pairwise removal option). A total of 729 positions were present in the last dataset. Evolutionary assessments were performed in MEGA X (Kumar et al., 2018).

ond division was mostly related to African honey bee species. The next subcluster contained of species mainly distributed in China and Saudi Arabia. But the third subcluster entailed species from Japan, Korea, Germany, and New Zealand. In this third sub-group of *A. mellifera*, one sequence (MN585108) from Ethiopia was very distant from all other specimens. *A. mellifera* sequence of Pakistan didn't fall into the group of *A. mellifera* but located near to a specimen (MF351881) from North Africa. Based on ND5 gene fragment, only two sequences of *A. dorsata* were found in the NCBI database and both sequences formed a single group. *A. dorsata* sequence of Pakistan was not related to previously submissions and fall in a separate group. *A. cerana* species also formed a single group but few species from Japan fall separately at larger distances. *A. cerana* sequence of Pakistan was also fell separately.

3.2. Genetic distance among honey bee species

Pairwise maximum distances based on COI gene fragment were computed among the *A. mellifera* sequence from Pakistan and the sequences from different countries obtained from the NCBI database. The overall maximum distance ranged between 0.00% and 0.024%. The maximum country-wise difference was seen equal to 0.023% between the *A. mellifera* population of Pakistan, Egypt, and South Africa whereas, the lowest country-wise divergence was observed up to 0.001% between the *A. mellifera* population of

Pakistan and London (Table S1). Pairwise maximum distances based on COI gene fragment were computed among the *A. cerana* sequence from Pakistan and the sequences from different countries obtained from the NCBI database. The overall maximum distance ranged between 0.000% and 0.073%. The maximum country-wise divergence was noted equal to 0.073% between the *A. cerana* population of Pakistan and Japan whereas, the lowest country-wise variance was witnessed equal to 0.048% between the *A. cerana* population of Pakistan and China (Table S2). Pairwise maximum distances based on COI gene fragment were computed among the *A. dorsata* sequence from Pakistan and other sequences from different countries obtained from the NCBI database. The overall maximum distance ranged between 0.001% and 0.078%. The maximum country-wise variance was equal to 0.071% between the *A. dorsata* population of Pakistan and Indonesia whereas, the lowest country-wise difference was up to 0.003% between the *A. dorsata* population of Pakistan and India (Table S3).

Pairwise maximum distances based on ND5 gene fragment were computed among the *A. mellifera* sequence from Pakistan and the sequences from different countries obtained from the NCBI database. The overall maximum distance ranged between 0.00% and 0.416%. The maximum country-wise difference was equal to 0.410% between the *A. mellifera* population of Pakistan, Germany and Algeria whereas, the lowest country-wise difference was equal to 0.402% between the *A. mellifera* population of Pakistan, the USA,

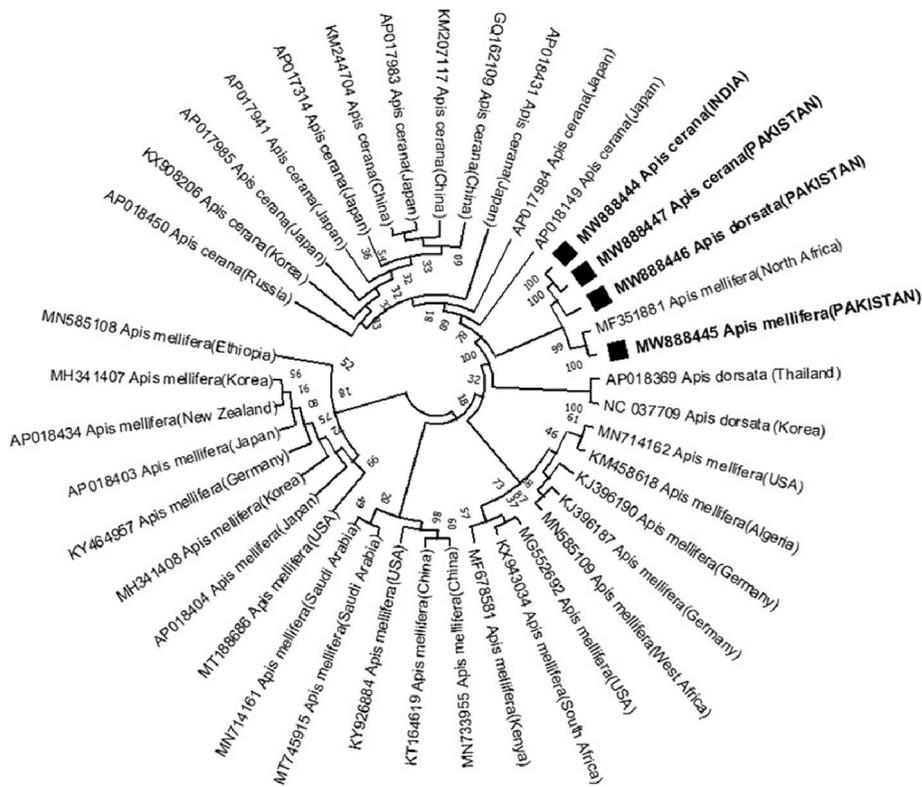


Fig. 2. The evolutionary track record was deduced engaging the Neighbor-Joining method (Saitou and Nei, 1987). The bootstrap unanimity tree extrapolated as of 1,000 repeats is shown to characterize the evolutionary record of the taxa examined. Outlets related to partitions produced under 50% bootstrap repeats are disintegrated. The proportion of repeat trees wherein the linked taxa grouped collectively in the bootstrap test (1,000 replicates) are displayed alongside the outlets (Felsenstein, 1985). The evolutionary spaces were computed employing the Kimura 2-parameter approach (Kimura, 1980) and are in the elements of the quantity of base replacements per site. This assessment engaged 40 nucleotide sequences. Codon positions engaged were first + second + third + Noncoding. The entire vague locations were deleted for every single sequence pair (pairwise removal option). A total of 1158 positions were present in the ultimate dataset. Evolutionary investigations were performed in MEGA X (Kumar et al., 2018).

Japan, Korea, Germany, Ethiopia and Saudi Arabia (Table S4). Pairwise maximum distances based on ND5 gene fragment were computed among the *A. cerana* sequence from Pakistan and the sequences from different countries obtained from the NCBI database. The overall maximum distance ranged between 0.000% and 0.046%. The maximum country-wise variance was 0.046% between the *A. cerana* population of Pakistan and China whereas, the lowest country-wise variance was 0.008% between the *A. cerana* population of Pakistan and India (Table S5). Pairwise maximum distances based on nd5 gene fragment were computed among the *A. dorsata* sequence from Pakistan and other sequences from different countries obtained from the NCBI database. The overall maximum distance ranged between 0.009% and 0.676%. The maximum country-wise difference was 0.676% between the *A. dorsata* population of Pakistan and Korea whereas, the lowest country-wise variance was seen up to 0.668% between the *A. dorsata* population of Pakistan and Thailand (Table S6).

3.3. Genetic diversity among honey bee species

Three honey bee species collected from Pakistan were compared with to their respective species belonging to different countries and their sequences were obtained NCBI database so, there ought to be certain genetic difference based on their geographical zones. The entire sequences were exposed to the genetic diversity evaluation, and diversity signs were assessed across two neutrality assessments, i.e., Fu and Li's F* and Tajima's D founded on pairwise nucleotide diversity. In COI gene fragment sequences, the highest genetic difference (built on Eta value) was noted in *A. dorsata* fol-

lowed by *A. cerana* showing up to 64 mutations and 43 mutations correspondingly, within the populations of different countries. In contrast, the *A. mellifera* population was the least diverse with 28 mutations. Similarly, In ND5 gene region sequences, the highest genetic variety (founded on Eta value) was seen in *A. mellifera* followed by *A. cerana* showing up to 337 and 266 mutations respectively, within the populations of different countries. While the *A. dorsata* population was the least diverse with 13 mutations. The importance of genetic variation was corroborated equally by neutrality tests that showed COI gene fragment sequences comparison, *A. cerana* had substantial difference built on the p-value of Fu and Li's assessment (0.02) and p-value of Tajima's D test (0.01), correspondingly. Instead, in ND5 gene fragment sequences comparison, *A. cerana* and *A. mellifera* had noteworthy variations built on the p-value of Fu and Li's test (0.02;0.02) and p-value of Tajima's D test (0.001; 0.001), separately. Neutrality tests of *A. dorsata* were not computed because four or more sequences were required and, in this study, we had only three sequences (Table 3). To validate all directories of genetic variety, entirely blasted sequences were assessed for population size deviations, to compute pairwise mismatch spreading for altogether sequences of different honey bee species. In general, and country-wise outcomes had articulated a substantial genetic difference for COI gene (Fig. 3) and ND5 gene (Fig. 4).

4. Discussion

Phylogenetic relationship based on COI gene sequences (both from this study and obtained from the NCBI database) has demar-

Table 3
Genetic variance computation of various honey bee species by DnaSP v5 via polymorphism data assessment.

Honey bee species	Gene	Sequences (n)	Nucleotide diversity						Neutrality test			
			Total number of sites	S	Eta	Pi	k	theta	Fu and Li's Fs	p-value	Tajima's D	p-value
<i>A. mellifera</i>	COI	NCBI = 23; PK = 1	631	28	28	0.0128	8.110	0.012	-0.430	>0.10	0.261	>0.10
<i>A. cerana</i>	COI	NCBI = 20; PK = 1	435	22	22	0.0081	3.526	0.014	-2.472	>0.10	-1.652	>0.10
<i>A. dorsata</i>	COI	NCBI = 18; PK = 1	470	57	64	0.0306	14.418	0.039	-1.056	>0.10	-0.935	>0.10
<i>A. mellifera</i>	ND5	NCBI = 22; PK = 1	708	331	337	0.0490	34.709	0.130	-4.381	<0.02	-2.558	<0.001
<i>A. cerana</i>	ND5	NCBI = 13; PK = 1	475	253	266	0.0925	42.589	0.186	-3.203	<0.02	-2.314	<0.001
<i>A. dorsata</i>	ND5	NCBI = 2; PK = 1	438	13	13	0.0311	13.000	0.031	2.565	0.929	N/A as four or more sequences are needed to compute Tajima's D	

Abbreviations: S = Number of variable sites, Eta = Total number of mutations, Pi = nucleotide diversity (per site), k = average number of nucleotide differences, theta = nucleotide substitution rate. Whereas a negative value of Fu's Li's Fs and Tajima's D test statistics is evidence for an excess number of alleles, whereas a positive is related to deficiency of alleles. Both Fu's Li's and Tajima's D test Fs were used to single nucleotide polymorphism.

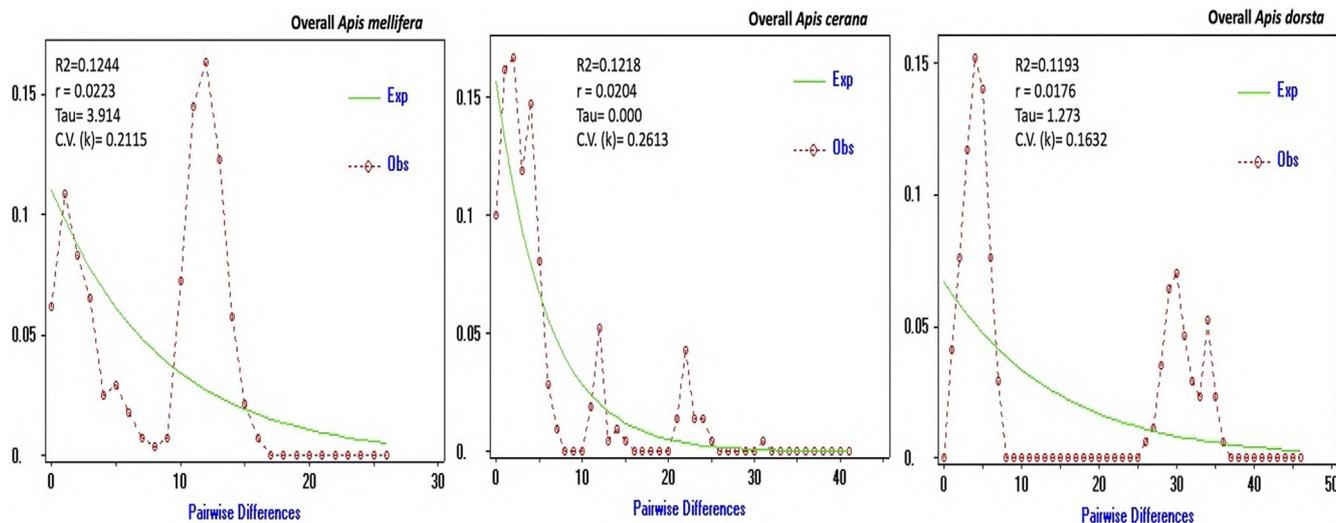


Fig. 3. Pairwise divergence allotments for three honey bee species, built on COI gene sequences with DnaSP v5. The X-axis reveals the noted allocation of pairwise genetic variant, and the Y-axis characterizes the rate of recurrence.

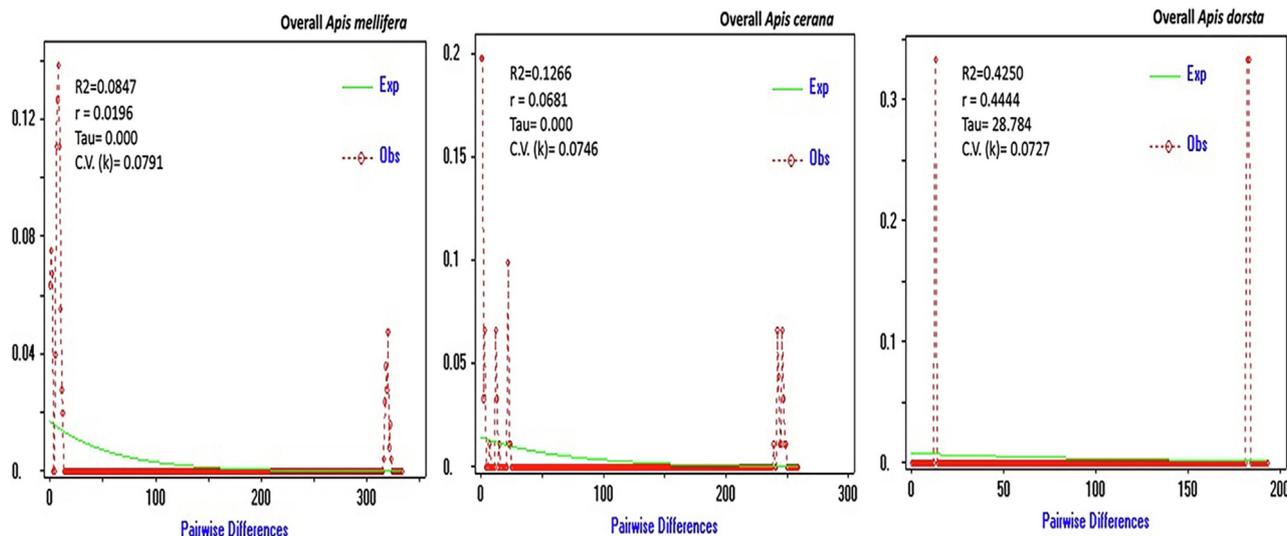


Fig. 4. Pairwise divergence allotments for three honey bee species, built on ND5 gene sequences with DnaSP v5. The X-axis reveals the noted allocation of pairwise genetic disparity, and the Y-axis characterizes the rate of recurrence.

cated the genetic collaboration amongst all honey bee species. The phylogenetic hierarchy of the genus *Apis* created on COI gene presented three main groups. All sequences of each species fall into their respective group, which are almost in accordance with Arias and Sheppard (2005) whose phylogenetic analysis reinforced the elementary topology recoverable as of morphometric study and assembling the honey bees hooked on three main groups of giant bees, dwarf bees, and cavity nesting bees. *A. mellifera* comprised three subgroups representing vast genetic interaction suggesting that mitochondrial genes represent high average genetic diversity which is in accordance with Ballard and Whitlock (2004) and Meemongkolkiat et al. (2019). Similarly, the phylogenetic relationship based on ND5 gene sequences (both from this study and obtained from the NCBI database) has defined the more genetic interaction among all honey bee species. The phylogenetic hierarchy of the genus *Apis* built on ND5 gene presented six main groups. *A. cerana* and *A. dorsata*, and one *A. cerana* from India fall into one sub-cluster of *A. mellifera*. Similar findings have been found previously when Engel and Schultz (1997) reanalyze the genus *Apis* constructed on morphological traits and available 16S sequence information after six taxa and they braced the elementary phylogeny of Lindauer (1956). In Lindauer phylogeny, two species (*A. mellifera* and *A. cerana*) that adopt cavity-nesting and make multiple combs were thought about the utmost newly derivatives and sister taxa. The clade of *A. mellifera* and *A. cerana* apportioned a joint ancestor by *A. dorsata* after that this consortium pooled to an ancestor through *A. florea*. The average number nucleotide difference of both gene fragments within and among the three honey bee species was low in COI gene fragment as compared to ND5 gene fragment. This suggests that there was little intra species variation but high inter species variations which is again in agreement with Ballard and Whitlock (2004) and Meemongkolkiat et al. (2019).

Garnery et al. (1991) reported that the ancestor species of the genus *Apis* diverged in two lineages spawning into open nesting single comb making species (*A. dorsata* and *A. florea*) along with cavity nesting numerous combs building species (*A. mellifera* and *A. cerana*) correspondingly. The Asian honey bees, *A. dorsata* and *A. florea* but then deviated quickly, the split of both species took place later. In this study, the specimen of *A. florea* was not investigated but the remaining results of the phylogenetic tree are on similar lines. The total number of nucleotide diversity in three honey bee species based on COI gene segment is in agreement with Garnery et al. (1991) who worked on phylogenetic interactions in the genus *Apis* deduced from mitochondrial DNA sequence data and reported the number of nucleotide exchanges detected in pairwise analogies and the subsequent distance based on CO-II gene. But the total number of nucleotide diversity based on ND5 gene segment is slightly higher than the results of Garnery et al. (1991). This depicted that ND5 gene offered more genetic diversity in the genus *Apis*.

The average number of nucleotide difference based on COI gene in *A. mellifera* was similar to Rizwan et al. (2018) who reported 7.772 for honey bee populations of Pakistan. Both *A. mellifera* used in this study and studied by Rizwan et al. (2018) exhibited massive genetic interaction and least diversity. This kind of genetic interaction was also noticed Martimianakis et al. (2011) who studied the phylogenetic interactions of the Greek honey bee population built on sequencing of mtDNA regions of COI and ND5 genes. They explained that different subspecies of *A. mellifera* form a relationship among them from diverse localities. These kinds of former studies witness our results of genetic interaction of *A. mellifera* population based on COI gene. While the average number of nucleotide difference based ND5 gene was higher within and

among the different honey bee species. The overall average number of nucleotide difference of mutually gene fragments in and among species was higher, particularly for ND5. This suggested variation within the genus *Apis*. Similar findings for ND2 mitochondrial gene were reported by Arias and Sheppard (2005) who suggested that in the genus *Apis*, the ND2 segment had the ability to solve the genetic affiliations amongst species. These findings are in accord with Abou-Shaara et al. (2021), Bouga et al. (2011), Meemongkolkiat et al. (2019), and Low et al. (2014) who suggested that mitochondrial genes are highly widespread molecular markers applied to ascertain the extent of genetic variations in insects including honey bees as the mitochondrial genome normally illustrates greater alteration rates. In the past different indices of genetic variations were evaluated up to species, genus, and family levels by many researchers to ascertain the genetic variation in the mitochondrial DNA. However, for mitochondrial DNA diversity in our samples of Asian and western honey bee species, mutations were calculated in COI and ND5 gene regions and compared with already reported honey bee sequences around the globe. A considerable genetic variation was observed between and among the honey bee species. In *A. mellifera* the genetic diversity was slight but based on ND5 gene sequences the variations were high. The possible reason may be that there are very few molecular studies on the Asian honey bee species.

5. Conclusion

The phylogenetic assessments of molecular information produced by sequencing of two distinctive mitochondrial regions (COI and ND5) generated consistent outcomes. Likewise, our results substantiated earlier statements in the scientific literature established upon molecular information and morphometric study to define the phylogenetic interactions in *Apis* species. Based on COI gene fragment in two Asian (*A. cerana* and *A. dorsata*), one European honey bee (*A. mellifera*) species from Pakistan, and 20 (*A. cerana*), 18 (*A. dorsata*), and 23 (*A. mellifera*) honey bee species sequences obtained from the NCBI data base around the globe showed considerable genetic variety indicators and deviation within and amongst species. While on the other hand based on ND5 gene fragment in two Asian (*A. cerana* and *A. dorsata*), one European honey bee (*A. mellifera*) species from Pakistan, and 13 (*A. cerana*), two (*A. dorsata*), and 22 (*A. mellifera*) honey bee species sequences obtained from NCBI data base around the globe showed comparatively high genetic diversity indices and variation than COI gene. This work shows the detailed genetic and phylogenetic interactions among the honey bee species found in Pakistan and other countries of the world. Inside the genus *Apis*, the mitochondrial ND5 area revealed an ability to solve the interactions among species. Within the genus *Apis*, a further detailed work by incorporating the evaluation of additional genomic and mitochondrial genes for the improved solution is required to further establish the concise genetic diversity and relationship among the species.

Declaration of Competing Interest

The author declares that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.sjbs.2021.07.062>.

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